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Bacillus atrophaeus spore viability following exposure to blast effects from the military explosive C4

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Comparison of *Bacillus atrophaeus* spore viability following exposure to detonation of C4 and to deflagration of halogen-containing thermites

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Energetic materials are being considered for the neutralization of spore-forming bacteria. In this study the neutralization effects of a monomolecular explosive were compared to the effects of halogen-containing thermites. *Bacillus atrophaeus* spores were exposed to the post-detonation environment of a 100 g charge of the military explosive C-4 at a range of 50 cm. These tests were performed in the thermodynamically-closed environment of a 506-liter barometric calorimeter. Associated temperatures were calculated using a thermodynamic model informed by calculations with the Cheetah thermochemical code. Temperatures in the range 2300-2800 K were calculated to persist for nearly the full 4 ms pressure observation time. After the detonation event, spores were characterized using optical microscopy and the number of viable spores was assessed. Results showed live spore survival rates in the range 0.01-1%. For the thermite tests a similar, smaller-scale configuration was employed that examined the spore neutralization effects of two thermites: aluminum with iodine pentoxide, and aluminum with potassium chlorate. Only the former mixture resulted in spore neutralization. These results indicate that the detonation environment produced by an explosive with no chemical biocides may provide effective spore neutralization similar to a deflagrating thermite containing iodine.

Introduction

Many defeat strategies for destroying potential stockpiles of viable spores are based on exposing the targeted biological material to explosively-generated high temperatures in addition to chemical biocides.[1, 2] Spores are susceptible to destruction by heating, even for sub-second durations and temperatures as low as 550 K.[3] Spores have also been shown to be susceptible to the chemical effects of biocides such as iodine, especially when generated in the gas phase.[4] It is difficult, however, to quantify the effectiveness of

explosive-based strategies for destruction of spores deposited on surfaces because of the violence of the test events and the generation of post-detonation debris which typically mixes with live and dead spores. The main objective of this study is to better understand the effects of detonation reactions on bacterial spores. A secondary objective is to compare the effects of detonation reactions with the effects of deflagrating thermite mixtures containing biocidal chemistry. This complementary approach addresses important issues related to the technical challenge of destroying biological agents.

Experimental

Experiments with detonating C4 were performed using a barometric calorimeter while complementary experiments with thermites were done in a Biocidal Reaction Chamber, as will be discussed in detail. We examine *Bacillus atrophaeus* (*B. atrophaeus*) as a surrogate for the pathogenic *Bacillus anthracis* because spores from the two species are biologically and structurally similar. *B. atrophaeus* has been widely used in related studies of mechanisms for spore destruction,[3-14] and so presents a useful species for our thermodynamically-controlled experiments.

Detonation Experiments: Barometric calorimeter description and experimental geometry

Experiments were performed in a 506-liter barometric calorimeter designed for characterizing the rate of energy release from explosive charges on time scales ranging from tens of microseconds to seconds.[15-17] The calorimeter consists of a closed cylindrical vessel with pressure gauges on the top surface. A schematic cross section of the calorimeter is shown in Figure 1(a). A Kistler 4075 piezoresistive pressure gauge placed in the top lid of the calorimeter, as shown in Figure 1(b), serves to demonstrate that the charge has fully detonated and to provide a pressure record which can be used as a basis for confirming subsequent thermodynamic temperature estimates.

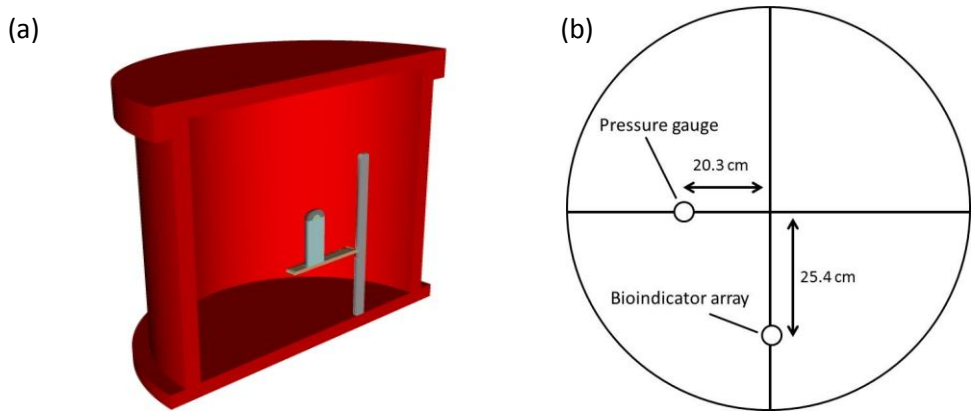

Fig. 1. (a) 506 liter barometric calorimeter model (red), with hemispherical charge on cylindrical Lexan pedestal in grey. The charge and Lexan pedestal are in turn supported from the base of the calorimeter by a metal stand, also illustrated in grey. (b) Top view of calorimeter indicating pressure gauge and sample holder locations for experiments.

Biological samples

Concave 9 mm diameter, stainless steel discs inoculated with 10^6 *Bacillus atrophaeus* subsp. *globigii* spores per disc, and packaged in individual Tyvek sleeves (also called Biological Indicators, BIs), were purchased from Apex Laboratories, Apex, NC. As previously noted, *B. atrophaeus* is a broadly-used surrogate for the pathogenic *Bacillus anthracis* bacterium. For each experiment, five BIs were carefully removed from their sleeve using sterile forceps, taking care not to contact the concave surface of the disc, and mounted into a custom-built sample holder. Sample handling used sterile microbiological techniques: all procedures were performed in a Class II BioSafety Cabinet using disposable tools and personnel protective equipment (gloves, forceps) for each sample to prevent any cross-contamination. Samples were transported to the experiment site using sterile, secondary containment, and the sample holder was then secured to the lid of the calorimeter. A typical pre-detonation BI surface is illustrated in Figure 2.

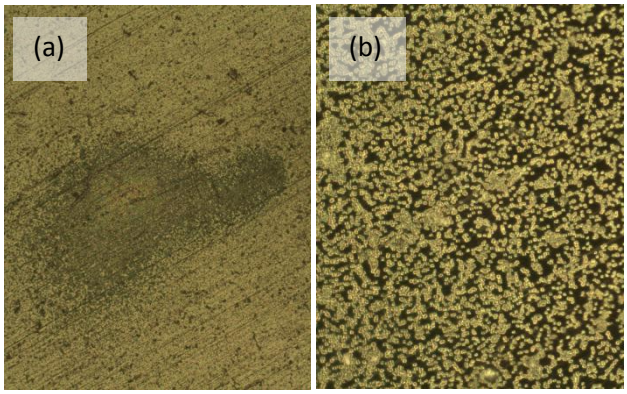

Fig. 2. (a) Optical microscope image of BI surface (5x objective) showing a clump of spores under bright field illumination conditions. (b) A magnified view of the BI surface (50x objective) showing spores under dark field illumination conditions.

These five BIs were then mounted on a fixture using sterile conditions; the fixture was connected to the top of the barometric calorimeter as shown in Figure 3(a), with the BI surfaces oriented toward the charge. The 100 g C-4 charge was positioned at the radial center of the barometric calorimeter, 43 cm from the circular walls. The charge was detonated with an RP-1 detonator, boosted by a 13 g hemisphere of LX-10. The charge was hemispherical, attached to a Lexan base, such that the bottom plane of the hemisphere was vertically centered in the calorimeter 43 cm from the top and bottom planar walls, as shown in Figure 4(b). The experiment was repeated three times.

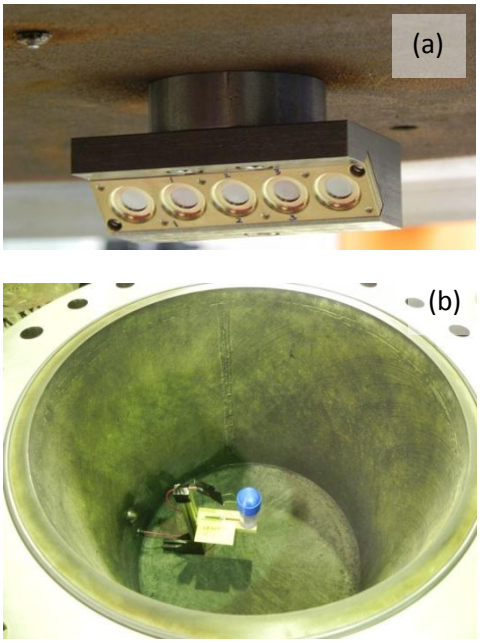

Fig. 3. (a) BI fixture installed in the lid of barometric calorimeter. With the lid in place, the BI surfaces directly face the hemispherical charge and are exposed to post-detonation products and temperatures. (b) 100 g C4 hemispherical charge positioned in the barometric calorimeter.

Deflagration Experiments: Exposing Spores to Halogen Based Thermite Reactions

The thermite experiments followed an identical protocol as previously described,[4] but will be summarized here. Two formulations were selected: aluminum with iodine pentoxide ($Al+I_2O_5$), and aluminum with potassium chlorate ($Al + KClO_4$). Both were prepared at a stoichiometry predicted to produce the highest adiabatic flame temperature. Loose powder mixtures of about 250 mg were placed in an enclosed Biocidal Reaction Chamber (BRC). The Bacillus atrophaeus spores were positioned about 12 cm from the thermite, as previously described.[4] Seven tests were performed for each thermite reaction that exposed four vials containing spore samples, such that 28 spore samples per thermite reaction were analysed for colony forming growth kinetics. Temperatures at the spore sample locations were measured using thermocouples. For both thermites, maximum temperatures did not exceed 50°C, with average temperatures ranging from 36-41°C.

Results

Explosive Testing: Effects on biological indicators (spore viability analysis)

Pressure records from each of the detonation experiments were very similar: pressures on the order of 1 MPa were recorded which persisted for at least 4 ms. After each experiment, BI

samples were analyzed to assess viable spore counts remaining after the detonation using a procedure for complex environmental samples. After the explosion, the sample holder was removed from the calorimeter and transported to the laboratory for microbiological analysis. Samples were carefully removed from the sample holder in a BSC using a new pair of gloves and new, disposable forceps for each BI, and transferred to individual sterile 30-mL glass tubes containing 10 mL of sterile, double-distilled water. In addition to the five samples exposed to the detonation, one negative control (sterilized BI) and one positive control (BI not exposed to the detonation) were added to the sample set for each experiment. Samples were left to incubate at room temperature for 15 min, followed by sonication in a water bath for 30 min. Three consecutive 10-fold dilutions were performed in phosphate buffered saline with 0.05% Tween 20, pH 7.4 (Teknova Inc., Cat. No. P0201) for each sample tube and plated on Tryptic Soy Agar in triplicate using the spread plate method. Plates were incubated at 30°C overnight, and counted the next day. All colonies observed on the plates presented the orange pigmentation associated with *B. atrophaeus*.

Table 1 summarizes the results obtained for a representative set of seven samples (the experiment was replicated, leading to similar results). No colony counts were observed for the negative control, confirming appropriate sample handling methods and the absence of cross-contamination. Colony counts obtained on the BI which was not exposed to the explosion were $5.9 \times 10^6 \pm 0.4 \times 10^6$, which confirmed the spore level inoculated on the BIs, as well as appropriate spore recoveries from the BI samples with our method. Colony counts obtained on BIs exposed to the detonation varied from $1 \times 10^2 \pm 1.7 \times 10^2$ to $5.3 \times 10^4 \pm 1.0 \times 10^4$ viable spores per BI, which corresponds to a 2- to 4-Log reduction in live spore population.

Table 1. Summary of microbiological analysis conducted on biological indicators before and after the explosion

Sample Type	**Colony Counts[1] (CFU/Sample)**
Negative Control[2]	0
Positive Control[3]	$5.9 \times 10^6 \pm 0.4 \times 10^6$
Biological Indicator 1	$4.3 \times 10^2 \pm 1.5 \times 10^2$
Biological Indicator 2	$5.3 \times 10^4 \pm 1.0 \times 10^4$
Biological Indicator 3	$1 \times 10^2 \pm 1.7 \times 10^2$
Biological Indicator 4	$1.4 \times 10^4 \pm 0.3 \times 10^4$
Biological Indicator 5	$2.8 \times 10^4 \pm 0.3 \times 10^4$

[1] Colony counts are corrected from dilution factors and are the average of three replicate plates. [2] Sterile biological indicator. [3] Biological indicator not exposed to the explosion.

We note that the viability assay only enumerates live spores located on the BI surface, and that this assay alone therefore does not provide all the information needed to reconstitute the entire spore population of the BI. For example, viable spores aerosolized and dispersed by the detonation would not be enumerated by the assay. To provide a more complete view of the post-detonation BI sample population, live/dead spore staining experiments were performed.

Effects on biological indicators: optical microscopy

During two baseline experiments, BIs were pre-positioned such that only a fraction of the spore-covered surface was exposed to the detonation. This allowed for measurement of spore viability across the boundary from shielded to unshielded surface. Five of these partially-exposed BIs are shown in Figure 4.

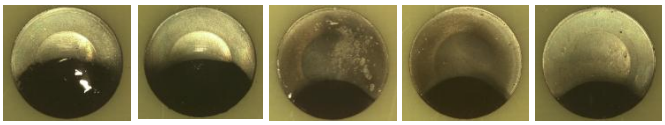

Fig. 4. Photographs of partially-exposed BIs. Dark regions are soot-covered areas which are further examined microscopically as shown in Figure 5 and Figure 6.

Micrographs of the region near the interface are shown in Figure 5. Agglomerates of carbon particles are visible on the surface, together with some spores.

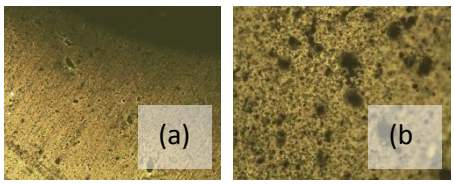

Fig. 5. Partially-exposed BIs (5x objective, left and 50x objective, right), near the shielded boundary.

Additional imaging was performed using a Zeiss Axiovert 200M microscope equipped with epifluorescence capability (Figure 6). All images were acquired with 50× magnification and a 1.6× Optovar lens for a total magnification of 80×. Three images were collected and merged for each location: darkfield, fluorescein isothiocyanate (FITC) excitation/emission and Texas red

(TR) excitation/emission. Spores were stained using Invitrogen's BacLight live/dead stain kit for 15 minutes following the manufacturer's instructions.

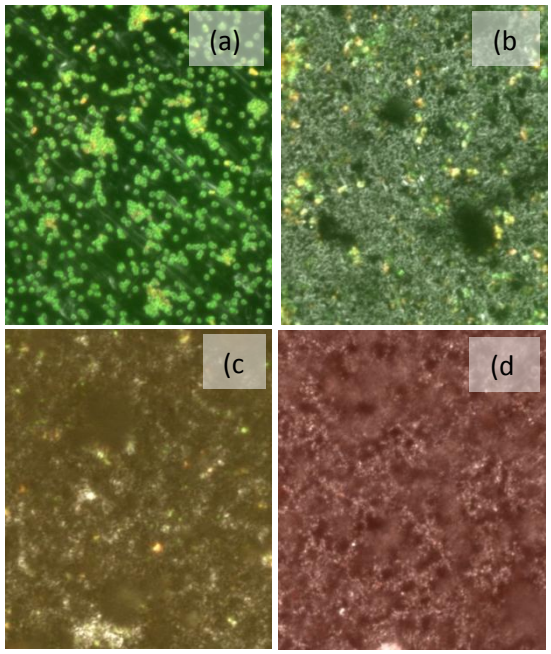

Fig. 6. Epifluoresce microscopy images of BI samples pre- and post-detonation. Red spores are compromised (non-viable), and green spores are viable. (a) No detonation control; fluorescein isothiocyanate (FITC): 19 s exposure (weak signal), Texas red (TR): 1.6 s (strong signal). (b) Post-detonation, soot-free area; FITC: 32 s exposure, TR: 5 s. (c) Post-detonation, moderate soot area; FITC: 32 s exposure, TR: 31 s. (d) Heavy soot area; FITC: 32 s exposure, TR: 32 s.

Figure 6(a) is a control image taken of a BI surface not exposed to the post-detonation environment. Figure 6(b) indicates the presence of both live and dead spores in a relatively soot-free area near the BI fixture border, where spores would have been subjected to limited detonation temperatures and pressures. Figure 6(c) also shows both live and dead spores interspersed with soot particles in a moderate soot area, where spores were more exposed to post-detonation conditions. Figure 6(d) shows that the heaviest soot deposits prevent imaging of any spores. Fluorescence microscopy images of stained spores embedded in soot revealed spores killed by post-detonation temperatures, consistent with the overall interpretation that the experimental conditions tested in this study led to the partial killing of *B. atrophaeus* spores.

Deflagration Testing: Quantifying Spore Neutralization

Quantifying spore neutralization was achieved in a similar manner presented previously for visually counting the number of colony forming units (CFU) that develop from a single viable spore during an incubation period.[4] A non-exposed control sample was processed alongside the exposed samples to provide a reference for growth kinetics. The data were collected from photographs taken during the incubation period. An imaging software program marked each colony counted, and an average growth count was determined.

For $Al+I_2O_5$ a mean CFU of 0.5 was measured, consistent with results presented for an identical mixture.[4] However, for $Al+KClO_4$ a mean CFU of 155 was measured. These results indicate that while the $Al+KClO_4$ reaction produce chlorine, the dispersion of this halogen during reaction does not reach nor effectively neutralize the spores. Because the temperature at the location of the spores does not exceed 50°C, only the chemical biocide could be responsible for spore neutralization. However, the biocide must exist in the gas phase and come in contact with the spore to be effective.

Discussion

Spectroscopic measurements of C-4 fireballs have indicated sustained temperatures in the range 2200-2500 K.[18-20] Because the barometric calorimeter represents a thermodynamically closed system, we can directly calculate temperatures associated with measured pressures. For example, the full complement of thermodynamic states have been calculated starting from the expanded detonation products gases of C-4, and the combustion of these products in air, as determined by the Cheetah thermochemical code. The constant volume detonation temperature for C-4 is 2887 K. In the case of adiabatic combustion, these gases and combustion products behave as an ideal gas below about 3000 K.[21] Therefore, measured pressures may be assumed to be directly proportional to temperature, which leads to a time-averaged value somewhat less than 2887 K over the 4 ms measurement time. A similar relationship has been calculated for TNT combustion products, where it was shown that temperatures in the range 2400-2700 K persist over 2-

10 ms in a constant volume explosion at a comparable fuel loading (mass/chamber volume).[22]

We can use simplifying assumptions to develop bounds on the rate at which spores may be heated by conduction in the post-detonation environment. A typical *Bacillus atrophaeus* spore can be approximated by an ellipsoid, with diameter 0.65 microns and length 1.2 microns.[23] The density of these spores (and most *Bacillus* species spores) is ~1.2 g/cm^3, close to the density of water.[24] Assuming one-dimensional planar thermal conduction, the temperature increase in the spore is exponentially dependent on the heating time, with a time constant given by:

$$t = d^2 \rho C_v / 4K \qquad (1)$$

where d is the spore diameter, ρ is the spore density (g/m^3), C_v (J/g K) the specific heat, and K (J/s m K) the thermal conductivity of the spore. For a given temperature differential, $T_{ambient} - T_{spore,\ initial}$, between spore and ambient, t is the time in seconds for the spore to reach a temperature equal to $(1\text{-}e^{-1}) \times T_{ambient} \approx 0.62 \times T_{ambient}$ (=1420 K for $T_{ambient}$ = 2300 K, for example).

Although the fundamental thermal properties of spores are difficult to measure directly, it is possible to obtain a range for the relevant temporal order of magnitude for spore heating by substituting the physical constants of the bounding cases of spores composed of either water or carbon in the diamond phase. For water $\rho = 10^6$ g/m^3, C_v = 4.18 J/g K, and K = 0.58 J/s m K; t is then 7.6×10^{-7} s . For diamond $\rho = 3.5 \times 10^6$ g/m^3, C_v = 0.51 J/g K, and K = 895 J/s m K, and t = 2.1×10^{-10} s. Both times are much shorter than the measured duration of the elevated post-detonation temperature, which is greater than 4×10^{-3} s. The water-derived heating time is consistent with *in situ* spectroscopically-measured decay rates associated with aerosolized *Bacillus atrophaeus* spore breakup in a shock tubes: 10^7 s^{-1} for temperatures in the range 1000 K, as measured by Gates et al.[3] We conclude that spores which are not thermally coupled to the substrate or other larger

thermal mass equilibrate with the ~2300 K post-detonation ambient gases in less than a millisecond.

The Gates et al. shock tube study demonstrates aerosolized spores of *Bacillus atrophaeus* physically break up when exposed to temperatures above 700 K for times shorter than 2.5×10^{-3} s. Viable fractions of the number of spores exposed to 1000 K for 2.5×10^{-3} s are in the range 10^{-5}. At temperatures of 550 K for the same amount of time, viable fractions increase to 10^{-1}.[3] Aerosolized spores exposed to flame heating have also been demonstrated to be efficiently destroyed in fractions of a second.[25] These data suggest that any spores aerosolized from the BI surface would rapidly be destroyed. The presence of live spores on the BI surface observed in these experiments is then likely due to efficient thermal coupling of spores to the stainless steel surface, or to much larger soot particles, where the greater thermal masses of these structures enable spores to remain at temperatures below 550 K for the duration of the detonation event.

Similar to the thermite results presented here, Clark and Pantoya previously examined the effect of thermites with and without biocidal chemicals, and found that even 250 mg quantities of an iodine-containing thermite could reduce viable dry *Bacillus atrophaeus* spore densities by more than 100x at a range of 12 cm.[4] Two biocidal thermites were investigated: aluminum combined with iodine pentoxide (I_2O_5), and Al combined with silver oxide (Ag_2O)). Al combined with iron oxide (Fe_2O_3) was also tested as a control; this mixture generates a highly exothermic reaction, but has no biocidal properties. Temperatures produced by the thermites at the location of the spore samples were shown to have little efficacy because of the microsecond reaction times and relatively short (i.e., approximately 12 cm) distance between thermite and spore. As in the results shown here for chlorine, the biocidal element silver was similarly ineffective, likely because both species were not transformed by the reaction into a gas phase that could be efficiently delivered to the spores. As demonstrated by others, it may be possible for biocidal silver to become more mobile if combined with iodine in a thermitic reaction of $Al/AgIO_3$, evolving into AgI.[26]

Conclusions

Detonation experiments demonstrate that in an aerobic confined environment, 100 g of C-4 in a 506 liter volume creates temperatures of at least ~2300 K persisting for several milliseconds. These conditions lead to *B. atrophaeus* spore survival rates in the range 0.01 to 1% on BI surfaces and therefore effective neutralization of the spores at a range of 50 cm. Fluorescence microscopy images of stained spores embedded in soot revealed spores killed by detonation temperatures, confirming that experimental conditions lead to the partial killing of *B. atrophaeus* spores on surfaces, as opposed to complete displacement and aerosolization of the spores. Spores displaced and aerosolized from the bioindicator surface and into the ~2300 K gas-phase ambient would be rapidly destroyed. The measured survival rates are comparable to those demonstrated with 400× smaller quantities of an iodine-containing thermite, aluminum/iodine pentoxide, at a range of 12 cm. These experiments provide a path toward a more quantitative and systematic assessment of explosively-generated effects for the destruction and sterilization of spores on surfaces in confined spaces.

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